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THE ROYAL VETERINARY AND AGRICULTURAL UNIVERSITY
Department of Clinical Studies

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hatting-KS□De Danske Kvægavlsforeninger	Examiner:	Sandra Saucier
Serial #:	09/914,765	Group art unit:	1651
Filed:	6 March 2000	Docket:	
Title:	Determination of sperm concentration and viability for artificial insemination		

DECLARATION BY Preben Christen and Torben Greve

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Torben Greve, DVM, DVSc, DVSc, hc. Professor of animal reproduction, do hereby declare as follows:

I, Preben Christensen, DVM, Associate Professor, PhD. The Royal Veterinary and Agricultural University, Department of Clinical Studies, Section for Reproduction, do hereby declare as follows:

I am one of the inventors of the above-identified application.

I have been engaged in the field of semen evaluation, particularly for use in the artificial insemination industry since 1992 and worked with the use of flow cytometry since 1995.

It is known in the art that the total number of sperm cells and the proportion of live sperm cells are important factors for achieving optimal fertility. It has been found that there seems to be a cut-off value below which the fertility drops below a certain percentage thus being below the profitable fertility rates. The total number of sperm cells and the proportion of live sperm cells required for obtaining this minimum acceptable fertility is different for different species.

The precision with which the total concentration of sperm cells and the proportion of live sperm cells are determined are of great importance, since a precise determination of these values provides

- the possibility of evaluating single ejaculates on the basis of a single determination,
- the possibility of using ejaculates having values closer to any predetermined cut-off value, since a more precise determination means that the safety distance to any predetermined cut-off value may be chosen according to the precision with which the determination is performed.

The simultaneous determination of the total concentration of sperm cells and the proportion of live sperm cells from a single sample increases the precision of the measurement in that the possibilities of mistakes and errors during handling and pipetting are reduced. In the present invention only one sample is prepared for determination whereas the prior art methods uses more determination routines and more measurements on samples prepared differently to obtain the total concentration and the proportion of live cells. Hereby, the sources of errors when handling and pipetting the samples and when measuring the number of samples are increased and the variation on the resulting determinations are thus equally increased.

Whatever methods are used for determination of the total sperm concentration separate from the determination of the proportion of live sperm cells, the variation will necessarily be larger than by combining the determinations in a single determination.

For the production of semen doses, it is of great importance to be able to select the ejaculates carefully so that for example ejaculates potentially providing a too small litter size may be rejected before insemination so as to increase the overall mean litter size produced. The present invention provides a more precise result due to the simultaneous determination of total concentration and the proportion of live sperm cells in each ejaculate and thus allows for rejection/acceptance of single ejaculates.

The precise determination of total concentration and number of living sperm cells obtainable according to the present invention makes it possible to go closer to the cut-off value for individual species / males / ejaculates and thereby produce a higher number of insemination doses.

By using the method of the present invention to determine total sperm concentration and proportion of live sperm cells and evaluate the semen quality on the basis of these determinations, a significant increase in insemination doses may be obtained. For example for boars, the increase in insemination doses may be up to 40 % compared to traditional methods.

These unexpected results of the simultaneous determination of total concentration and proportion of live sperm cells are not disclosed in any of the references mentioned by the Examiner. The fact that each individual ejaculate may be evaluated on the basis of a single measurement instead of having to use the mean value of several measurements on the same or a number of ejaculates do provide the unexpected possibility of selection of valuable ejaculates for insemination and maximizing the number of semen doses that can be produced from an ejaculate.

A number of experiments has been carried out, one of them with the aim of assessing if flow cytometric determination of sperm concentration and viability could result in a more uniform production of semen doses. Results of this experiment showed high precision in the determination of sperm concentration and coefficients of variation were 3.5 % and 2.4 % for raw and frozen-thawed semen, respectively. Sperm viability was also assessed with high precision and coefficients of variation were 0.9 % for raw semen and 1.7 % for frozen-thawed semen. Furthermore, the experiment showed that package of semen doses after flow cytometric determination of sperm concentration in the raw semen results in a significantly smaller variation in the number of sperm per dose

Preparation of Sperm Counting Reagent and dilution of semen samples

The Sperm Counting Reagent was supplied by BD Biosciences Immunocytometry Systems (San Jose, CA). The sealed polyethylene counting tubes were initially vortexed for 5 sec in up-right position and 5 sec inverted before opened. The dye solution in the kit contained SYBR-14 and propidium iodide (PI) dissolved in DMSO and addition of 20 µl to a counting tube resulted in final concentrations of 50 nM SYBR-14 and 12 µM PI. All counting tubes were prepared with dyes on the same day of use (< 12 h prior to use). After preparation as well as prior to use, counting tubes were vortexed for 5 sec (only up-right position).

Analysis on the FACSCount AF flow cytometer

Excitation with the FACSCount AF flow cytometer is performed with a fiber-coupled 488 nm external air-cooled laser. The instrument collects two parameters of fluorescence and one parameter of size data for each event. Signals were separated by a 620 nm short pass dichroic mirror. The green fluorescence was collected through a 515 - 545 nm band pass filter while the red fluorescence was collected through a 645 nm long pass filter. All signals were recorded on a 4-decade logarithmic scale and no compensation was used. Subsequent data analysis was performed automatically by the flow cytometer without interference from the operator and results of the analysis was displayed on a print-out (Christensen et al, 2003).

Experimental design

Experiment 1: Semen production

In each of the four bull studs, 72 ejaculates were collected and processed during 6 experimental days. Sperm concentration in the raw semen was assessed by use of the Sysmex F-820 electronic cell counter (Sysmex GmbH, Hamburg, Germany) in stud 2 while the three other studs (1, 3 and 4) used the L'Aiglon spectrophotometer (IMV, Cedex, France). All assessments were performed by two labtechnicians in studs 2, 3 and 4, while only one labtechnician participated for stud 1. Motility assessments were performed at 200 x magnification using phase contrast and a heated stage (37°C). For motility assessments, a sample of the raw semen was diluted 1:10 dilution in the Triladyl freezing extender (Minitüb, Tifenbach, Germany) containing 20 % v/w egg yolk. Prior to flow cytometric analyses, a sample of the raw semen was diluted 1:250 in CellWash (BD Biosciences Immunocytometry Systems, San Jose, CA) and 50 µl of this dilution was transferred to a counting tube using a FACScount pipette (BD Biosciences Immunocytometry Systems, San Jose, CA). Incubation was for 4 min at room temperature. At the beginning of the incubation as well as prior to analysis, the tube was vortexed briefly (2 sec). The flow cytometric analyses were carried out in duplicate.

On each experimental day, four ejaculates were assigned at random to one of three treatment groups. Treatment A was according to the previous procedure for production of bull semen and the quantity of the ejaculate in this group was measured by reading the volume in ml with one decimal in a cylinderglas. For treatment B, the weight of the ejaculate was determined on an electronic scale and the volume was calculated using an average density of 1.07 g/ml. In treatment groups A and B, the dilution of raw semen prior to packing in straws was based on measurement of sperm concentration by Sysmex F-820 electronic cell counter or the L'Aiglon spectrophotometer as mentioned above. For treatment group C, sperm concentration was determined by the FACSCount AF flow cytometer and the volume of the ejaculates was based on the weight. All the procedures for determination of quantity and quality of the ejaculates were performed in the same order to avoid biased

results. The weight of the ejaculates was determined after the visual assessment, and the flow cytometric analyses (which also determine the percentage of live sperm) were performed after the microscopic assessment of sperm motility. After packing and freezing procedures, two straws from each batch were thawed and motility was assessed as described above. After motility assessments, a sample of the frozen-thawed semen was diluted 1:20 in a phosphate buffered saline (PBS, pH = 7.4) and 50 µl was transferred to a counting tube and stained for 5 min. The flow cytometric analyses were carried out in duplicate.

Statistical analysis

Data from experiment 1 were subjected to descriptive analysis and comparison of means and standard deviations were carried out with the Student's t-test.

Results

Experiment 1: Semen production

The analyses with the FACSCount AF flow cytometer were performed in duplicate to assess the precision of the system when operated by labtechnicians. The average coefficient of variation was 3.5 % for assessment of sperm concentration in raw semen and 2.4 % for assessments performed on frozen-thawed semen. For assessments of the sperm viability, the average coefficient of variation were 0.9 % and 1.7 % for respectively raw and frozen-thawed semen.

Results for semen production in the three treatment groups are shown in Table 1. The target was to package approximately 15 x 10^6 total sperm per straw which corresponds to 65.2 x 10^6 sperm/ml (straw volume = 0.23 ml). Treatments A and B differed slightly from this target (respectively 73.3 and 70.2 x 10^6 sperm/ml) while straws packaged according to flow cytometry in combination with estimation of ejaculate volume from the weight (treatment C) averaged 62.3 x 10^6 sperm/ml. A significant difference in mean sperm concentration was observed between the different treatment groups, with treatment group C closest to the target. Also the smallest variation in sperm concentration was observed for semen batches packaged according to treatment C (standard deviations respectively 11.5 x 10^6 (treatment A) and 13.1 x 10^6 sperm/ml (B) versus 6.5 x 10^6 sperm/ml (C), both $P<0.0001$).

Table 1. Sperm concentration in straws packaged according to three different treatments by four different bull studs. The values express the sperm concentration in 10^6 sperm/ml (means ± standard deviation). All results are based on post-thaw determinations of sperm concentration with the FACSCount AF flow cytometer.

	Treatment A	Treatment B	Treatment C
Bull stud 1	74.3 ± 9.2	80.4 ± 10.8	63.5 ± 5.5
Bull stud 2	74.2 ± 9.2	59.8 ± 13.0	61.8 ± 6.8
Bull stud 3	76.5 ± 16.2	71.2 ± 15.2	60.2 ± 7.3
Bull stud 4	68.3 ± 11.4	69.4 ± 13.1	63.5 ± 6.3
Total	73.3 ± 11.5[a]	70.2 ± 13.1[a]	62.3 ± 6.5[b]

Treatment A: Straws were packaged according to estimation of sperm concentration in the raw semen by spectrophotometer or electronic cell counter in combination with visual estimation of ejaculate volume.

Treatment B: Sperm concentration was determined as for treatment A, but ejaculate volume was determined from the weight.

Treatment C: Sperm concentration was determined with the FACSCount AF flow cytometer and ejaculate volume was determined from the weight.

Standard deviations (SD) differed significantly ($P<0.0001$) if superscripts after the SD's are different.

Semen production in experiment 1 showed that the standard deviation for sperm concentration in straws form different batches of semen could be reduced from 13.1×10^6 sperm/ml (treatment B) to 6.5×10^6 sperm/ml when sperm concentration in the raw semen was determined by FACSCount AF. This difference in standard deviation is highly significant ($P<0.0001$) and corresponds largely to a halving of the 95% confidence interval for the number of sperm per straw. Given that a certain minimum number of sperm per dose is required for optimal fertility (den Daas et al, 1998, Amann and Hammerstedt, 2002), the average target for the number of sperm per dose can be reduced significantly by use of the FACSCount AF flow cytometer. We have estimated that the production straws due to the improved precision can be increased by approximately 15 to 20 %.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statement and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United State Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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month/day/year

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month/day/year

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Christensen, P., Stenvang, J.P., Godfrey, W. A flow cytometric method for rapid determination of sperm concentration and viability in mammalian and avian semen. J. Androl. 2004, 25:255-264.

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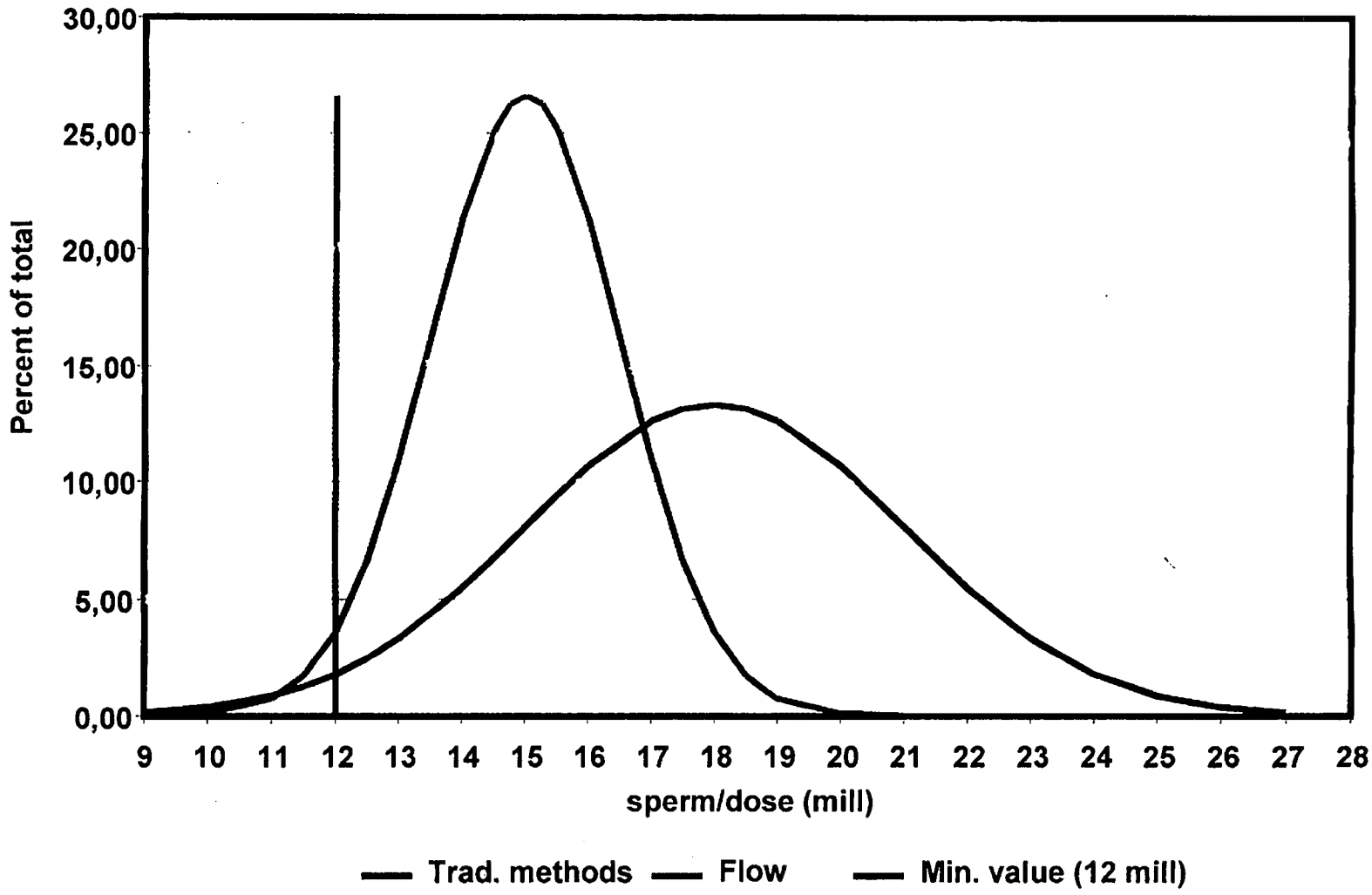
Production of spermdoses



Rapid Determination on Sperm Cell Concentration in Bovine Semen by Flow Cytometry[1]

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ABSTRACT

A flow cytometric technique is described for determining sperm concentration in fresh or extended semen with improved accuracy, precision, repeatability, ease of conduct, and rapidity. The technique is designed to measure the ratio of a known number of fluorescent beads admixed with sperm stained with either acridine orange or propidium iodide. A significant advantage of the technique is the distinct resolution between sperm and other particles (e.g., somatic cells, fat droplets, and bacteria in the semen or extender) that interfere in other counting protocols. Field testing of this protocol over the past 3 yr has demonstrated its superiority over the Coulter counter, hemacytometer, and spectrophotometer for accuracy in counting sperm in extended semen and the accuracy of counting sperm in straws based on preextension spectrophotometric determination of sperm concentration. Sperm chromatin quality can be determined simultaneously with this sperm counting procedure. This approach to counting sperm provides an excellent procedure for quality control of sperm numbers in processed semen.

(**Key words**: sperm count, flow cytometry, fluorescent beads, deoxyribonucleic acid stains)

Abbreviation key: α_t = the ratio of red fluorescence to total fluorescence, **AO** = acridine orange, **[CC]** = sperm concentration determined by Coulter counter, **[CFC]** = sperm concentration determined by flow cytometry based on Coulter counter determination of stock fluorescent bead concentration, **%COMP**α_t = percentage of cells outside the main population of α_t, **EAIC** = Eastern Artificial Insemination Cooperative, Inc., **FC** = flow cytometry, **[Goal]** = predicted sperm concentration in straws, **[HEMA]** = sperm concentration determined by hemacytometer, **[HFC]** = sperm concentration determined by flow cytometry based on hemacytometer determination of stock fluorescent bead concentration, **PI** = propidium iodide, **SCSA** = sperm chromatin structure assay, **SD**α_t = standard deviation of α_t.

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INTRODUCTION

Accurate determination of sperm cell concentration is critical to the AI industry because it provides assurance both to bull studs and to customers that straws of extended semen contain the sperm numbers indicated. An accurate measure of sperm concentration is particularly important in export markets in which verification of numbers may be required. Routine sperm counts can help to identify possible processing errors within a specific batch of semen or on a particular day, should those errors occur. As sperm counting procedures become more refined, routine counting can be used to monitor subtle changes in daily semen processing that might affect the number of sperm packaged in a straw.

Hemacytometers are widely used for routine sperm counts, but the equipment is slow, and multiple measurements of each sample are needed. Single hemacytometer counts are not highly accurate; because of inherent errors in the technique, Freund and Carol (13) found that mean differences of 20% were not uncommon between duplicate sperm count determinations by the same technician. Electronic counters provide much more rapid counting, are easier to use, and give more repeatable results among technicians. However, those instruments tend to include in the sperm count any somatic cells present, immature sperm forms, cytoplasmic droplets, debris, and bacteria, thereby inflating the concentration value (19). Parks et al. (16) developed a detergent treatment that improved sperm resolution during electronic cell counting by eliminating interfering particles in extended semen.

Currently, the primary method used by the AI industry to estimate sperm concentration is spectrophotometric determination of turbidity of a semen sample using an instrument previously calibrated for sperm concentration with a hemacytometer or Coulter counter (1). This approach is only as accurate as the methods used for spectrophotometer calibration.

New, more accurate methods for sperm count determinations are being sought to replace the older ones. Some laboratories are trying the Makler™ counting chamber (Seif-Medical, Haifa, Israel) and other improved hemacytometers, such as the Microcell™ (Fertility Technologies, Inc., Natick, MA); however, these techniques will likely have problems similar to those associated with the standard hemacytometers. Takacs et al. (18) described counting sperm using light scatter on the flow cytometer. Ax et al. (1) described a method that involves staining diluted semen with the DNA fluorescent dye Hoescht 33258 (Sigma Chemical Co., St. Louis, MO) and relating measured amount of DNA fluorescence in a fluorometer to a standard of stained calf thymus DNA. The technique is of interest, but any other cells present have a 10-fold higher stainability than sperm (8), which can cause a very significant error in estimation of the number of sperm.

We present herein an accurate, reliable, and technically straightforward approach to counting sperm using a flow cytometer. Additional advantages over existing techniques are that this approach is faster than the hemacytometer and that cellular debris, fat droplets, and other particulate material in extended semen are not erroneously counted as sperm, as often occurs with electronic cell counters. The basis for this procedure is to stain selectively the sperm head with a DNA fluorescent stain [i.e., acridine orange (AO) or propidium iodide (PI)] and then to mix the labeled sperm with a known concentration of fluorescent beads. Fluorescent beads with the necessary purity and uniformity in size and shape for use with sperm are commercially available. The number of fluorescing particles is determined using the flow cytometer, and the ratio of fluorescent beads to sperm cells is calculated. Sperm concentration can then be calculated based on the previously determined bead concentration and the sperm to bead ratio. This technique has been used for counting somatic cells obtained from tissue biopsies (17). A critical aspect of the method is the precise and accurate determination of fluorescent bead concentration in the working suspension using either a hemacytometer or Coulter counter. Evenson and Ballachey (7) have made a preliminary report on this technique, and its application to routine quality control during semen processing has been reported (15).

This study considered the practicality of flow cytometry (FC) measurements made on different days, using different bead dilutions, different ratios of sperm to fluorescent beads, and independent measurements of fluorescent bead concentrations using a hemacytometer

and a Coulter counter. We also assessed whether our standard sperm chromatin structure assay [SCSA; (6, 9)] on sperm quality could be performed at the same time as determination of sperm concentration.

MATERIALS AND METHODS

Methodology at South Dakota State University

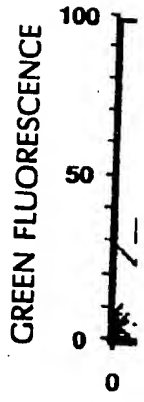
Bead Preparation. Stock solutions of fluorescent beads were prepared from concentrated, commercially available 25% Bright Fluorospheres (Coulter Electronics, Inc., Hialeah, FL). An aliquot of the fluorospheres was diluted 10-fold using a calibrated autopipette (400 to 1000 μl ± 1%) with TNE buffer (10 m*M* Tris, .15 *M* NaCl, and 1.0 m*M* EDTA; pH 7.4) measured with a glass volumetric pipette. Fluorescent bead concentration of the stock suspension was determined by both hemacytometer and Coulter counting. Before each stock solution was prepared, the 25% Bright Fluorosphere bottle was subjected to 2 min of sonication in a water bath sonicator (Branson model 32; Branson, Inc., Shelton, CT) and then vortexed (Vortex Genie™; Scientific Industries, Inc., Bohemia, NY) vigorously to obtain a well-dispersed, single bead suspension. Also, the stock solution used for mixing with semen was sonicated again for 2 min if it sat for 1 h or more.

Semen Preparation. Milk extender was used for all processed semen in these studies unless specified otherwise. Straws of frozen semen were thawed, and the semen was extruded into a 5.0-ml plastic test tube. Using a 2.0-ml glass volumetric pipet (1.998 ± .013 g of H_2O), 2.0 ml of TNE buffer were added to a separate 5.0-ml round bottomed plastic test tube. Using an Eppendorf™ pipet of ±.5% accuracy (Eppendorf-Netheler-Hinz-GmbH, Hamburg, Germany), 100 μl of TNE buffer were removed and discarded, and 100 μl of semen were added. To prepare the sample for a combined analysis of sperm chromatin structural integrity and sperm count, 100 μl each of the stock fluorescent bead suspension (vortexed for 3 s at top speed) and diluted sperm suspension were combined and stained with AO for the SCSA.

Staining Procedure and SCSA. For the SCSA, 200-μl aliquots of sperm cells and fluorescent beads were mixed with 400 μl of .1% Triton X-100, .15 *M* NaCl, and .08*N* HCl (pH 1.4). After 30 s, the cells were stained by adding 1.2 ml of a solution containing 6 μg of chromatographically purified AO (Polysciences, Inc., Warrington, PA)/ml of AO buffer [370 ml of .1 *M* citric acid, 630 ml of .2 *M* Na_2HPO_4, 1.0 m*M* EDTA, and .15 *M* NaCl; pH 6.0 (5, 10)].

Triton X-100 was used in the first step of the staining procedure to permeabilize the cell membranes, providing accessibility of DNA for AO staining. When excited by blue laser light, AO intercalated into native, double-stranded DNA fluoresces green (F_{530}); AO associated with single-stranded nucleic acid (DNA or RNA) emits red fluorescence ($F_{\geq 600}$). Because normal, mature sperm cells contain virtually no RNA (14), red fluorescence is minimal (12). Abnormal chromatin structure, defined as an increased susceptibility to acid- or heat-induced denaturation, is determined by FC measurements of the shift from green (native DNA) to red (denatured, single-stranded DNA) fluorescence. This shift is expressed by α_t (4), the ratio of red to total (red plus green) fluorescence. Measurement of normal sperm produces a very narrow α_t distribution, whereas that of sperm with denatured DNA is broader. Standard deviation of α_t ($SD\alpha_t$) describes the extent of chromatin structure abnormality within a population. Percentage of cells outside the main population of α_t (%$COMP\alpha_t$) represent the percentage of cells in the population with abnormal chromatin that are likely infertile (2, 3, 9).

FC Measurements. For each sample, 10,000 fluorescent particles (i.e., fluorescent beads plus stained sperm cells) were measured using an approximate sperm:bead ratio of 10. Samples were measured in a Cytofluorograf™ II flow cytometer (Ortho Diagnostics, Inc., Westwood, MA) equipped with ultrasense optics and a Lexel 100-mW argon ion laser operated at 35 mW with an excitation wavelength of 488 nm. To obtain the number of fluorescent beads and sperm in a sample, regions were gated on the computer screen around the respective fluorescent bead and sperm populations in the green versus red fluorescence cytogram (see Figure 1). Calculation of concentration = (sperm count ÷ bead count) × dilution factors × fluorescent bead stock concentration.



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Methodology a

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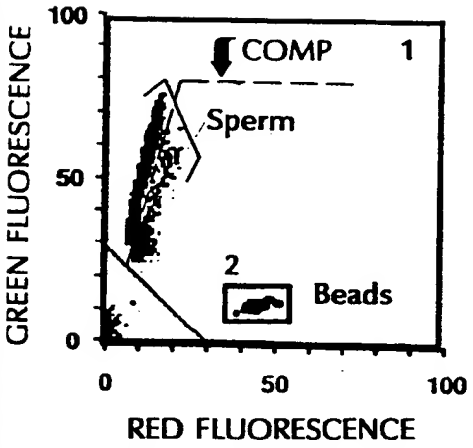

Figure 1. Green versus red fluorescence cytogram of acridine orange-stained, milk-extended semen and admixed fluorescent beads. Box 1 excludes debris in lower left hand corner and includes sperm and beads. Box 2 includes fluorescent beads alone. COMP = Cells outside the main population.

Methodology at Cornell University

Staining Procedure. Frozen semen containing between 40 and 80 × 10^6 sperm/ml was thawed and diluted 1:99 (vol/vol) with a Hamilton™ diluter (Hamilton Co., Reno, NV; 100 μl of extended semen plus 9.9 ml of TNE buffer containing .1% Triton X-100). A 400-μl aliquot of fluorescent beads (25% Bright Fluorospheres, 10 μm in diameter; Coulter Corp.) was diluted 1:24 (vol/vol) with TNE buffer using a 1000-μl Pipetman™ (Gilson Instruments, Woburn, MA) and Hamilton™ diluter (9.60 ml). The diluted fluorescent bead concentration used was approximately 8 × 10^5 beads/ml as determined with a Coulter Counter Z_m. Aliquots (200 μl each) of sperm and fluorescent beads were transferred using a 200-μl Pipetman™ into 1.5-ml microfuge tubes, and PI stain was added at a final concentration of 12.5 μg/ml. The stock PI solution was made up at 1 mg/ml in H_2O and stored at 4°C in a glass vial wrapped in aluminum foil. Samples were thoroughly vortexed immediately prior to counting. The sperm:bead ratio was determined, and the sperm concentration was calculated by multiplying the sperm:bead ratio × dilution factors. Sperm concentration determined by FC based on Coulter counter determination of stock fluorescent bead concentration [CFC] was divided by the predicted concentration from initial extension to arrive at a percentage of goal.

FC Measurements. An EPICS Profile FC (Coulter) was used to quantify sperm and fluorescent beads. Propidium iodide, a fluorescent DNA probe, and fluorescent beads were excited at 488 nm using an argon ion laser at 15 mW of power. Fluorescence emission was measured using a 457- to 502-nm laser blocking filter and a 515-nm long pass filter to block unwanted laser scatter, a 550-nm short pass dichroic mirror and a 525-nm bandpass filter to detect bead fluorescence, and a 590-nm long pass filter for PI detection. Fluorescence of the beads was also detected with the PI fluorescence detector but was off the scale with respect to the PI fluorescence. Analysis regions were set around the sperm and fluorescent bead populations for counting. Aliquots of 50 μl were analyzed.

RESULTS

FC Resolution Among Sperm, Fluorescent Beads, and Debris

AO Fluorochrome. Figure 1 shows raw data of FC measurements (Cytofluorograf™ II) of an approximate 1:100 ratio mixture of fluorescent beads and AO-stained bull sperm in milk-extended semen. The scattergram (cytogram) shows very clear resolution among sperm, fluorescent beads, and debris in the lower left corner. For determination of sperm concentration, the debris is gated out from the data set, and computer computation of the ratio of beads to total sperm is determined. This semen had a subpopulation of sperm with abnormal sperm chromatin structure, which have an increased susceptibility to DNA denaturation and a correspondingly increased red fluorescence. This abnormal population is gated off by the box labeled COMP, which is an acronym for cells outside the main population. Given the generally high quality of semen from commercial bull studs, the great majority of samples have low levels of %COMPα_t, and, thus, the scattergram dots representing these cells are rarely in the region of the fluorescent beads. If such a sample were analyzed, some loss of

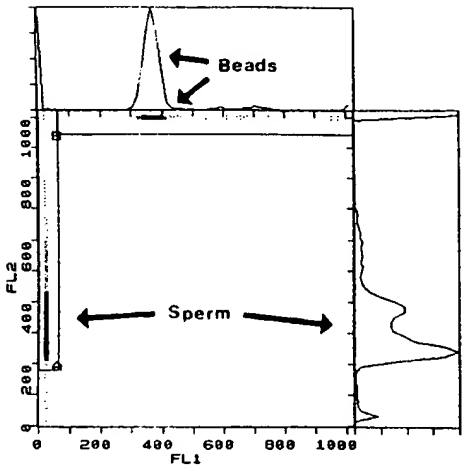

Figure 2. Dual parameter flow cytometry (EPICS profile; Coulter Electronics, Hialeah, FL) frequency histograms showing three populations: sperm stained with propidium iodide, fluorescent beads, and debris (lower left corner). FL1 = Green fluorescence, FL2 = red fluorescence.

accuracy in the FC sperm count determination would occur. Because the SCSA provides a sensitive means to detect alterations in sperm chromatin structure and because changes have been related to subfertility (2, 3, 9) and environmental stress (6), there are advantages in using the SCSA in combination with fluorescent beads to measure sperm count and sperm chromatin integrity simultaneously. One other advantage is that AO staining allows the cluster of dots representing sperm to be farther away from the Y-axis of the cytogram, thereby allowing easier resolution of sperm from potential debris.

PI Fluorochrome. Figure 2 shows dual parameter (green versus red fluorescence) data obtained from FC measurements (EPICS Profile) of PI-stained bull sperm in suspension with fluorescent beads. The three peaks corresponding to sperm, fluorescent beads, and debris (lower left corner) are gated for determination of the ratio of sperm:beads.

Optimal Range of Sperm:Bead Ratio for Accurate Counting

Serial dilution data, obtained from more than 10 experiments in which 1) the sperm concentration was held constant and the fluorescent bead concentration was varied and 2) the fluorescent bead concentration was held constant and the sperm concentration was varied, demonstrated that the method was very precise and repeatable over a broad range of sperm:bead ratios. As an example, shown in Figure 3, the FC-derived sperm count had no significant deviation from theoretical over the range of 1 fluorescent bead:100 sperm to 1 sperm:100 fluorescent beads. This wide range provides practicality for evaluating semen samples of unknown concentrations.

Sperm Concentrations Determined by FC and Hemacytometer at South Dakota State University with Coulter Counting at Cornell University

All milk-extended semen was processed and frozen in .5-ml polyvinyl chloride straws by personnel at Eastern Artificial Insemination Cooperative, Inc. (EAIC, Ithaca, NY). The predicted sperm concentration in the straws was calculated from initial spectrophotometric determination of sperm concentration in the undiluted semen, hereafter referred to as the goal concentration ([Goal]).

Sperm concentrations were determined on milk-extended semen samples that were also analyzed at Cornell University using a Coulter counter. Semen was packaged at EAIC with different [Goal]. The FC measurements were made, in random order and over several days, on 3 semen straws each from 32 collections out of 25 bulls. Duplicate or triplicate measurements were made on each straw, yielding 196 unique observations [32 collections × 3 straws = 96 × 2 (in some cases × 3) = 196]. The [CFC] and sperm concentrations determined by FC based on hemacytometer determination of fluorescent stock bead concentrations ([HFC]) were used. Six fluorescent bead stock solutions were used over the course of the measurements; these solutions had average concentrations of 2.450 (± .1378) × 10^5 and 2.399 ± (.0969) × 10^5, respectively, determined by hemacytometer and Coulter counter. Sperm concentrations measured by the Coulter counter ([CC]) at Cornell University were generally measured on 1 straw without duplication. Sperm concentrations determined by hemacytometer ([HEMA]; n = 8) were also

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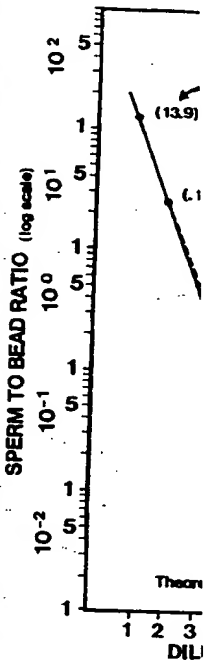

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The [HFC] and [CFC] for all 196 observations had mean (± SD) values of 5.4163 (± 1.3468) × 10^7 and 5.2638 (± 1.2779) × 10^7, respectively; correlation coefficient was .9893. Both FC counts are slightly lower than [CC] and [HEMA] (Table 1). Correlation coefficients of [HFC] and [CFC] with the [Goal] set by EAIC were .9683 and .9678.

Figure 4 shows simple regression (n = 18) lines on [HFC], [HEMA], [CC], and [Goal] regressed on [CFC]. The R^2 values were .9813, .9543, .9218, and .9211, respectively.

The correlations between $SD\alpha_t$ and [HFC] and [CFC] for the 25 bulls were .36 ($P < .01$) and .38 ($P < .01$).

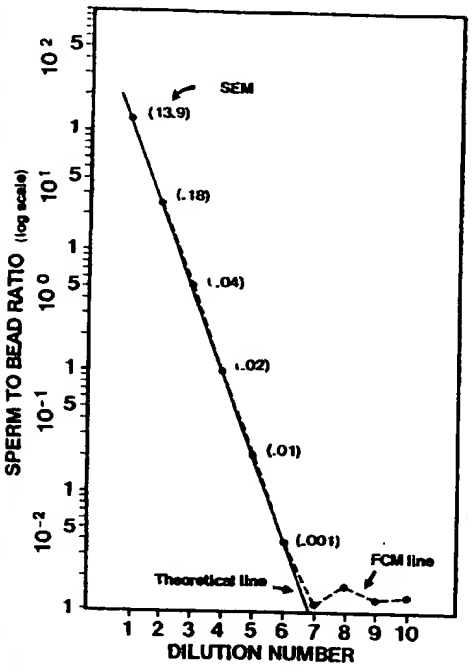

Figure 3. Comparison of theoretically measured with flow cytometry-measured sperm to fluorescent bead ratios of 10 samples with fluorescent bead concentration held constant and sperm serially diluted. Sample 1 had a sperm concentration of 11 × 10^6/ml and a sperm:bead ratio of 125:1. Samples were stained with acridine orange and measured in a Cytofluorograf II flow cytometer. The SEM for dilutions 7 to 10 were .001, .003, .003, and .002, respectively.

Cornell University Studies on Fresh Semen

Sixty-seven fresh ejaculates were collected on 4 different d and counted using a hemacytometer, Coulter counter, and EPICS Profile FC. Hemacytometer and Coulter counts were done in duplicate. Two FC concentrations ([CFC 1] and [CFC 2]) were measured in duplicate for each of 2 bead concentrations. The average of these two [CFC] was regressed on [HEMA] and [CC].

Means for sperm concentration determined by different procedures and correlations between the different counting procedures are presented in Table 2. No difference was found between [CFC] using different bead concentrations. The R^2 values from simple regression of average [CFC] on [HEMA] and [CC] were .9434 and .9851, respectively.

Relationship Between Predicted and Measured Sperm Concentration in Processed Semen

The [CFC] was determined in straws of milk-extended semen processed to contain between 40 and 80 × 10^6 sperm/ml. These [CFC] were lower than, but closer to, [Goal] than were routine [CC]. The variation in FC counts also tended to be slightly less than [CC]. When 2 straws were measured independently from 196 collections, no differences were measured in [CFC] among straws, and variation was equivalent for first and second straws measured. These results are presented in Table 3 for each [Goal] in the trial and indicate that, for the semen evaluated, percentage of [Goal] was slightly lower for semen packaged at 40 × 10^6 sperm/ml ($P < .05$) than for that packaged at higher concentrations. No explanation for this slight difference is obvious, but it is not likely due to concentration. At higher [Goal], [CFC] was 98 to 100% of the predicted concentration.

DISCUSSION

Flow cytometric determination of the ratio of a known concentration of fluorescent beads admixed with semen samples provides the basis for a very rapid and highly accurate method of determining sperm concentration in fresh or extended semen samples. This method can also be used for simultaneous assessment of sperm chromatin integrity; the SCSA provides an in-

TABLE 1. Means and correlations of sperm concentration determined by different procedures.

Concentrations	(n)	$\bar{X}$	SD	Range
			(× 10^7)	
[HFC][1]	32	5.3822	1.3527	3.6385–7.8601
[CFC][2]	32	5.2312	1.2932	3.5839–7.6716
[CC][3]	30	5.7527	1.5649	3.5400–8.8200
[Goal][4]	30	5.1185	1.2952	3.7450–7.4910
[HEMA][5]	18	5.9533	1.4626	4.0400–8.8000

	Correlation coefficients			
	[CFC]	[CC]	[Goal]	[HEMA]
[HFC]	.9976	.9715	.9683	.9774
[CFC]		.9710	.9678	.9806
[CC]			.9713	.9635
[Goal]				.9652

[1]Sperm concentration determined by flow cytometry based on hemacytometer bead count.

[2]Sperm concentration determined by flow cytometry based on Coulter counter bead count.

[3]Sperm concentration determined by Coulter counter.

[4]Predicted sperm concentration in straws based on initial spectrophotometric determination in undiluted semen.

[5]Sperm concentration determined by hemacytometer.

dex of susceptibility of sperm DNA to denaturation, which has been correlated with fertility (3, 6, 9) and exposure to toxic agents (10). Both AO and PI dyes provide an assessment of increased DNA staining because of immature sperm; this phenomenon has not been seen often in bull semen from commercial bull studs but is frequently seen with human semen (11) and occasionally with stallion semen (Evenson et al., 1993, unpublished data). This method can also be used to determine the number of somatic cells in a semen sample. Somatic cells (e.g., leukocytes) have 10-fold greater stainability with DNA dyes such as PI and AO than sperm, and both cell types can be measured with linear scale FC data processing (12); however, a log-scale amplification may allow easier resolution.

Although [HFC] and [CFC] were highly correlated, the Coulter counter is recommended for measuring bead concentrations because of greater speed and accuracy. However, if an electronic particle counter is not readily available, the hemacytometer is suitable, provided that a number of independent measurements (e.g., 10 loadings) are made.

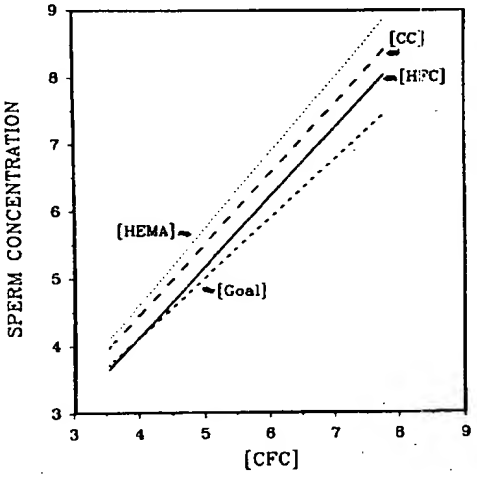

Figure 4. Simple regression lines of [HFC], [HEMA], [CC] and [Goal] regressed on [CFC], where [HFC] = sperm concentration determined by flow cytometry based on hemacytometer determination of stock fluorescent bead concentration, [HEMA] = sperm concentration determined by hemacytometer, [CC] = sperm concentration determined by Coulter counter, [Goal] = predicted sperm concentration in straws, and [CFC] = sperm concentration determined by flow cytometry based on Coulter counter determination of stock fluorescent bead concentration.

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[CFC 2][2]
[CFC, avg][3]
[CC][4]
[HEMA][5]

[CFC1]
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TABLE 2. Means and correlations of sperm concentrations measured by different procedures on fresh ejaculates of semen at Cornell University.

Concentrations	(n)	$\bar{X}$	SD	Range
				(× 10^9)
[CFC 1][1]	67	1.2739	.6767	.0780–2.8069
[CFC 2][2]	67	1.2739	.6751	.0740–2.7238
[CFC, avg][3]	67	1.2739	.6748	.0760–2.7262
[CC][4]	67	1.3270	.6940	.0836–2.8280
[HEMA][5]	67	1.3014	.6655	.0735–2.8500

	Correlation coefficients			
	[CFC 2]	[CFC, avg]	[CC]	[HEMA]
[CFC1]	.9932	.9983	.9685	.9906
[CFC2]		.9983	.9708	.9910
[CFC, avg]			.9713	.9925
[CC]				.9760

[1]Sperm concentration determined by flow cytometry based on hemacytometer bead count, bead solution 1.

[2]Sperm concentration determined by flow cytometry based on hemacytometer bead count, bead solution 2.

[3]Average flow cytometric sperm concentration.

[4]Sperm concentration determined by Coulter counter.

[5]Sperm concentration determined by hemacytometer.

Both [HEMA] and [CC] are slightly but consistently higher than sperm concentrations derived by FC in both studies in which they were compared. Measurement of nonsperm particles with the Coulter counter likely accounts for the differences in [CC]. Hemacytometer counts are subject to error, but these should be random and neither high nor low.

In our original study, we noted that, in one-third of the cases for which a repeat measurement was different by about 10%, a break in time during measurement was involved. Our original routine procedure was to place the stock fluorescent bead solution in a sonication bath every hour and then to vortex the solution prior to use before each sample. Because fluorescent beads appear to clump with time, the bead suspension should either be subjected to sonication prior to each sample or subjected to more vigorous vortexing.

The SCSA can be used in conjunction with samples that do not have severe abnormalities in chromatin structure. The samples of semen obtained from the AI industry that are from bulls highly selected for semen quality generally fit this category. Because of the low correlations between $SD\alpha_t$ and the [HFC] and [CFC] (.36 and .38), straws of lower semen quality apparently contained a larger number of sperm.

Virtually all quality control sperm counting done for EAIC at Cornell University since 1989 has used FC as described herein. Currently, triplicate straws from 10 batches of semen are counted each week. For each group of samples counted, 2 straws from one or more batches of semen held in storage are counted each week and serve as an internal control. All records are computerized and updated weekly and can be sorted by date, quality control

TABLE 3. Effect of [Goal][1] on sperm concentration determined by flow cytometry of processed semen as percentage of [Goal].

[Goal]	n	$\bar{X}$	SD	SE
		(%)		
40	56	94.4[a]	10.2	1.4
50	36	98.6	7.6	1.3
60	116	99.4	9.3	.9
70	14	100.1	6.4	1.7
80	164	98.1	9.5	.7

[a]$P < .05$.

[1]Predicted sperm concentration in straws (millions per milliliter).

number, bull code, straw, and [Goal]. This current procedure for counting sperm in milk extender is a significant improvement over previous procedures. The percentage of [Goal] using this procedure is much closer to the theoretical 100% than with detergent treatment and Coulter counting [98 vs. 113%, (16)], and variation is substantially reduced. Routine counting of sperm in semen that is extended, processed, and packaged in straws confirms that sperm numbers in straws accurately reflect the number predicted based on preextension determination of sperm concentration using the spectrophotometer. We conclude that the use of FC to determine sperm concentration in extended semen in highly accurate, is simple to perform, and circumvents many previous problems associated with sperm counting. This approach to sperm counting should be very useful in monitoring semen-processing procedures and confirming sperm concentration in semen processed for AI.

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